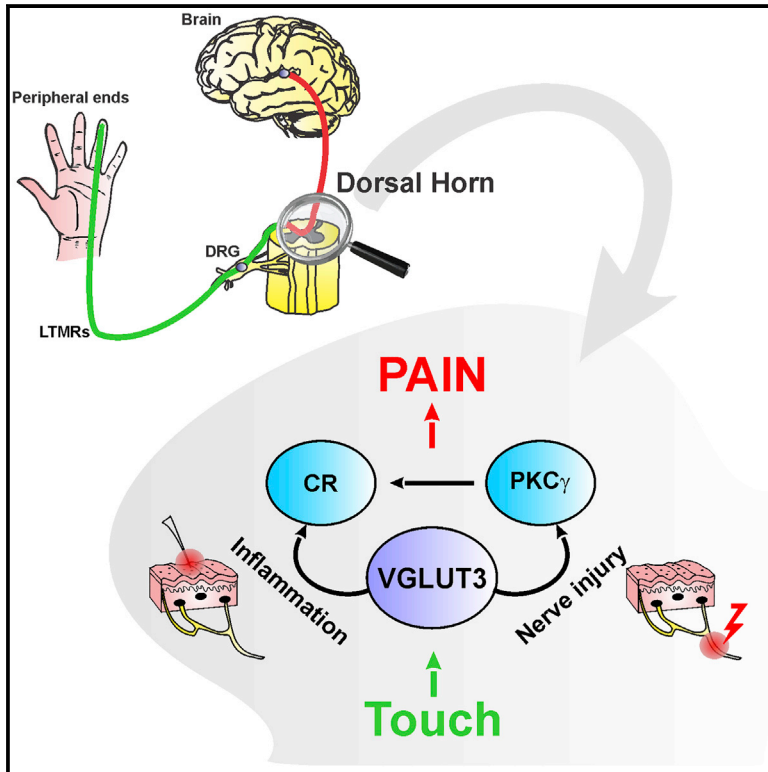


Neuron

Dorsal Horn Circuits for Persistent Mechanical Pain

Graphical Abstract



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In Brief

Peirs et al. identified deep dorsal horn neurons that transiently express VGLUT3 and are required for mechanical allodynia. The neurons transmit innocuous input to dorsally directed pain microcircuits that are differentially engaged depending on the type of injury.

Highlights

- Deep dorsal horn neurons transiently express VGLUT3 and convey mechanical allodynia
- They receive innocuous input and reside at the interface between touch and pain
- Their local activation reveals a dorsally directed pathway for mechanical allodynia
- Different microcircuits encode mechanical allodynia depending on the injury



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Dorsal Horn Circuits for Persistent Mechanical Pain

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SUMMARY

Persistent mechanical hypersensitivity that occurs in the setting of injury or disease remains a major clinical problem largely because the underlying neural circuitry is still not known. Here we report the functional identification of key components of the elusive dorsal horn circuit for mechanical allodynia. We show that the transient expression of VGLUT3 by a discrete population of neurons in the deep dorsal horn is required for mechanical pain and that activation of the cells in the adult conveys mechanical hypersensitivity. The cells, which receive direct low threshold input, point to a novel location for circuit initiation. Subsequent analysis of c-Fos reveals the circuit extends dorsally to nociceptive lamina I projection neurons, and includes lamina II calretinin neurons, which we show also convey mechanical allodynia. Lastly, using inflammatory and neuropathic pain models, we show that multiple microcircuits in the dorsal horn encode this form of pain.

INTRODUCTION

Mechanical pain conveyed acutely is beneficial, serving to warn the body of impending injury. However, persistent pain that results from injury or disease often becomes pathological and debilitating and is difficult to treat (Varrassi et al., 2010). Persistent pain states typically manifest as an increased sensitivity to thermal or mechanical stimuli. The latter allodynic state, in which innocuous touch or movement is perceived as painful, is one of the most clinically problematic forms of pain.

Persistent pain conditions are typically initiated by the dysregulation of primary sensory neurons leading to central sensitization within the spinal cord network and brain (Kuner, 2010; Prescott et al., 2014; von Hehn et al., 2012). The spinal cord dorsal horn is a major site of integration for somatosensory information and is composed of numerous excitatory and inhibitory interneuron populations and a relatively small number of output

neurons (Todd, 2010). Information processing in this structure is crudely segregated, such that primary afferents conveying thermal and nociceptive information mainly innervate the superficial laminae, whereas those transmitting low threshold mechanical information generally target deeper laminae. Almost all output neurons are located in laminae I and III-V. The vast majority of lamina I projection neurons respond to noxious stimuli and express the receptor for neurokinin I (NK1R). These cells are thought to convey discriminative aspects of pain, such as the location and quality, as well as emotional aspects (Miraucourt et al., 2007; Todd, 2010). In the deep dorsal horn, most projection neurons are wide-dynamic range. These neurons respond to many different types of stimuli, possess very large receptive fields, and code for stimulus intensity, among other features (Craig, 2003).

One model proposed for the circuit level mechanisms underlying mechanical allodynia is based on the gate control theory, in which touch normally inhibits acute pain through direct activation of inhibitory interneurons (Melzack and Wall, 1965). Upon injury, however, mechanisms of disinhibition allow touch to instead directly activate pain circuits. Experimental evidence consistent with this theory has demonstrated the existence of a dorsally directed polysynaptic pathway for mechanical pain that cannot be activated by innocuous mechanical input (touch) under normal conditions, due to a surrounding feedforward inhibition, or “gate” (Duan et al., 2014; Lu et al., 2013; Torsney and MacDermott, 2006). Upon injury, various mechanisms trigger disinhibition, thus opening the gate and allowing the low threshold mechanoreceptors to engage the polysynaptic network to activate nociceptive-specific projection neurons in lamina I (Baba et al., 2003; Miraucourt et al., 2007; Torsney and MacDermott, 2006; Zeilhofer et al., 2012). This model provides a framework to begin to understand the anatomical substrates and mechanisms underlying mechanical allodynia, but we still do not know many of the fundamental aspects of the circuit, such as the identity of the neurons involved or how the circuit is organized.

Vesicular glutamate transporters (VGLUTs) package glutamate into synaptic vesicles for regulated release. We previously reported that VGLUT3, which has a relatively sparse distribution in the nervous system, is required specifically for acute mechanical pain and the persistent mechanical pain that develops

in various models including inflammatory and neuropathic pain (Seal et al., 2009). All other somatosensory behaviors tested in mice lacking the transporter are normal, including thermal, itch, touch, and persistent heat hypersensitivity.

Here, we took advantage of the specific requirement for VGLUT3 in mechanical pain to explore the cellular basis of the circuit using a number of complementary approaches. From the analyses, we have now identified a discrete population of spinal cord excitatory interneurons as the origin of the mechanical pain defects in VGLUT3 knockout (KO) mice. Interestingly, the cells reside in lamina III, a region important for touch but largely ignored with respect to pain (but see Polgár et al., 2007a). We now also show that the cells participate in the persistent mechanical pain circuit and receive almost exclusively low threshold input, thus positioning them at a critical entry point to the circuit. We also identified additional excitatory populations that participate in the dorsal horn circuit for persistent mechanical hypersensitivity, including neurons in inner lamina II that express calretinin and were recently suggested to transmit only light acute mechanical pain (Duan et al., 2014). Lastly, we provide evidence that the neuronal composition of the circuit differs for different types of injury, indicating the existence of microcircuits for mechanical hypersensitivity. A better understanding of the microcircuits and their relationship to the injury will lead to more effective treatment strategies.

RESULTS

Characterization of Conditional VGLUT3 KO Mice

To identify the locus of the acute and persistent mechanical pain defects observed in global VGLUT3 KO mice, we used our floxed conditional KO line (VGLUT3^{fl/fl}) (see Figure S1A available online). Western blot analysis of brain lysates and immunohistochemistry performed on spinal cord slices from homozygous floxed mice indicate that VGLUT3 levels are not altered by loxP insertion (Figures S1B and S1C). We also produced a germline deletion, VGLUT3^{Δ/Δ}, by crossing VGLUT3^{fl/fl} to Rosa26^{Cre} mice. Biochemical and immunohistochemical analyses confirmed that this mouse line does not express the transporter (Figures S1B and S1C). Importantly, the VGLUT3^{Δ/Δ} mice show attenuated acute mechanical pain in the Randall-Selitto assay as well as significantly reduced mechanical hypersensitivity in both the carrageenan model of inflammatory pain and the spared nerve injury (SNI) model of neuropathic pain (Figure S1D), similar to VGLUT3 global KO mice (Seal et al., 2008, 2009).

Cellular Origin of the Mechanical Pain Defects in VGLUT3 KO Mice

In adult mouse spinal cord, VGLUT3 is largely restricted to a discrete population of dorsal root ganglion (DRG) neurons, the C-low threshold mechanoreceptors (C-LTMRs) that innervate dorsal horn lamina II (Figure S1C) (Seal et al., 2009). To assess whether mechanical pain sensation requires VGLUT3 expression by these cells, we deleted the transporter in all DRG neurons using Advillin^{Cre} mice (Figures 1A–1D). This line expresses Cre only in DRG and not spinal cord or brain (Figure 1A) (Hasegawa et al., 2007). Indeed, when the Advillin^{Cre} mouse was crossed to the Isl-tdTomato reporter (Madisen et al., 2010) all DRG neurons

expressed tomato (Figure 1B). As expected, VGLUT3 immunoreactivity was not detected in the dorsal horn of adult VGLUT3^{fl/fl}; Advillin^{Cre} mice (Figure 1C). Tail withdrawal thresholds of VGLUT3^{fl/fl}; Advillin^{Cre} mice did not differ from those of VGLUT3^{fl/fl} control mice (Figure 1D), indicating that loss of VGLUT3 from DRG does not affect acute mechanical pain sensation. In both the carrageenan and SNI models, the ipsilateral paw withdrawal thresholds of the VGLUT3^{fl/fl}; Advillin^{Cre} mice were similar to VGLUT3^{fl/fl} control mice and significantly reduced compared to the contralateral paw (Figure 1D), indicating that loss of VGLUT3 in DRG also does not affect mechanical hypersensitivity. Consistent with these results, loss of VGLUT3 from unmyelinated DRG neurons, including the C-LTMRs, using the SNS^{Cre} line (Agarwal et al., 2004) also did not alter mechanical pain behavior (Figures S1E–S1H).

Several brain regions implicated in pain processing express VGLUT3. Alternatively, dorsal horn neurons at early postnatal ages as well as Merkel cells, the end organ of slowly adapting mechanoreceptors, also express the transporter (Lou et al., 2013). To address whether VGLUT3 expressed by either of the latter two populations has a role in mechanical pain, we generated VGLUT3^{fl/fl}; Hoxb8^{Cre} mice (Figures 1E–1H). Hoxb8^{Cre} mice express Cre in all neurons that lie caudal to cervical levels 2–3, and not in the brain (Figure 1E) (Witschi et al., 2010). Immunoreactivity for VGLUT3 in the VGLUT3^{fl/fl}; Hoxb8^{Cre} mouse was absent from the lumbar dorsal horn, but still present in the striatum (Figures 1F and 1G). Withdrawal thresholds in both the acute and persistent mechanical pain assays were all markedly elevated in these mice compared to VGLUT3^{fl/fl} controls (Figure 1H). To delete VGLUT3 specifically in Merkel cells, we crossed the VGLUT3^{fl/fl} mice to the KRT14^{Cre} line (Figure 1I), a line that expresses Cre specifically in keratinocytes and Merkel cells (Figures 1J and 1K) (Hafner et al., 2004). Mechanical pain behavior in VGLUT3^{fl/fl}; KRT14^{Cre} mice was similar to control VGLUT3^{fl/fl} mice (Figure 1L). Taken together, these data point to dorsal horn neurons as the likely locus of the pain defects.

Dorsal Horn Neurons Underlie the Defects in Mechanical Pain

To examine the distribution of neurons that transiently express VGLUT3 in the dorsal horn, we crossed our BAC transgenic VGLUT3^{Cre} mice (Grimes et al., 2011) to the Isl-tdTomato mice. The resulting mice show both the adult and developmental expression of VGLUT3, as tomato is present in all cells that have ever expressed Cre. In the dorsal horn, tomato⁺ neurons are present in lamina III and to a lesser extent lamina II (Figure 2A). We also examined the dorsal horn of the VGLUT3^{EGFP} BAC transgenic line, in which EGFP expression is under the direct control of VGLUT3 regulatory elements (Figure 2B) (Seal et al., 2009). In this line, we observe a peak number of EGFP⁺ cells at postnatal day (P) 10–12, but the reporter is present only in the central terminals of C-LTMRs in the adult (Figure 2B). We also analyzed VGLUT3 expression in the dorsal horn directly by taking advantage of the VGLUT3^{fl/fl}; Advillin^{Cre} mice. Because VGLUT3 is deleted from primary afferents in these mice, expression is thus restricted to spinal cord neurons. Here we observed VGLUT3 immunoreactivity that first appeared around P5,

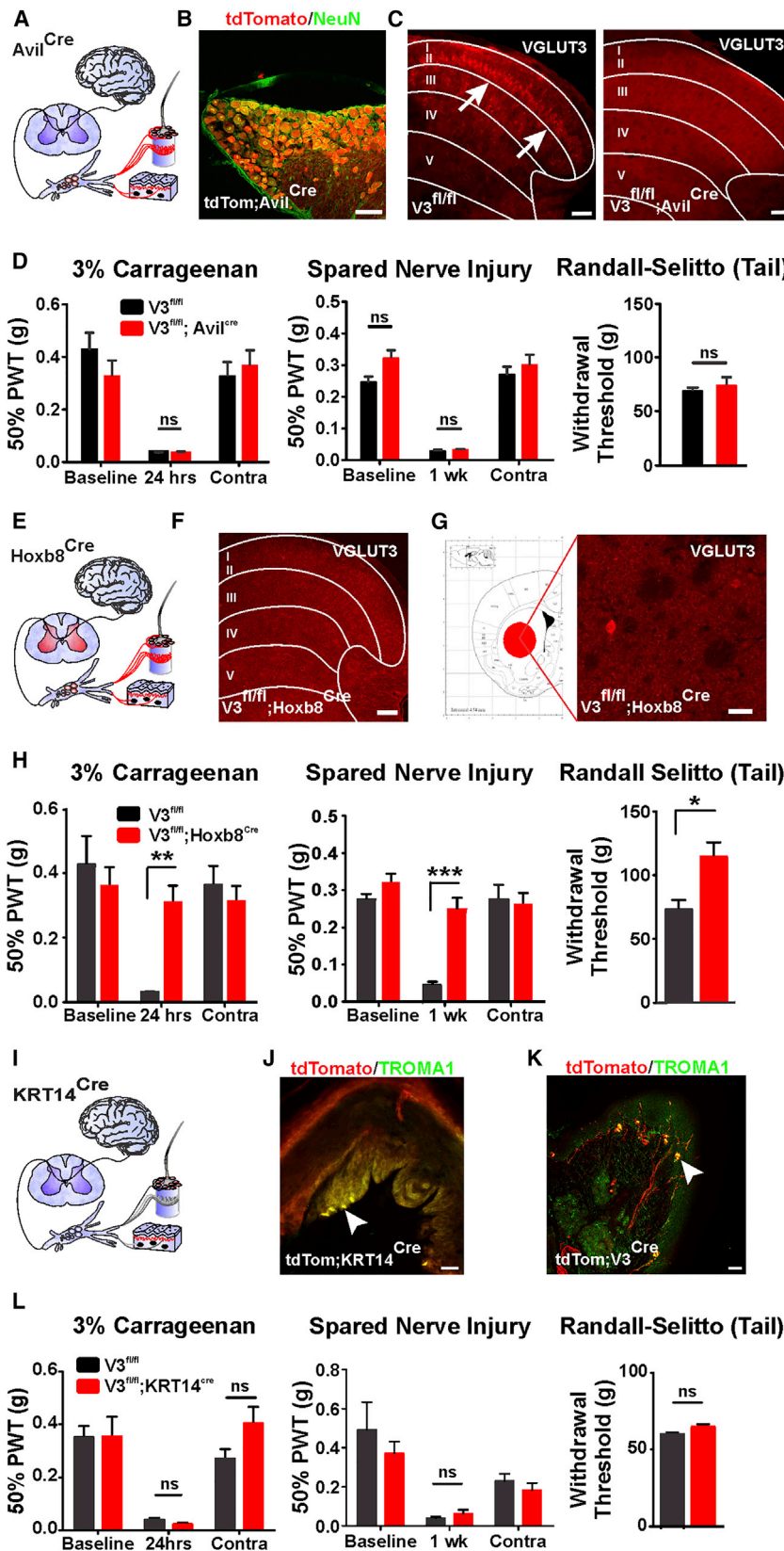


Figure 1. Mechanical Pain Defects Do Not Arise from DRG or Brain

(A) Cartoon of *Avil*^{Cre} mice showing Cre (red) is restricted to primary afferents.

(B) All DRG neurons express tomato in *Isl*-*tdTomato*; *Avil*^{Cre} mice.

(C) VGLUT3-IR is not detected in primary afferents (arrows) of *VGLUT3*^{fl/fl}; *Avil*^{Cre} in the adult.

(D) Paw withdrawal thresholds (PWTs) of *VGLUT3*^{fl/fl}; *Avil*^{Cre} mice do not differ from control mice after carrageenan ($n = 12$, both groups) or SNI ($n = 7$ and $n = 9$, respectively). Randall-Selitto thresholds also do not differ from controls ($n = 8$ and $n = 6$, respectively).

(E) Cartoon of *Hoxb8*^{Cre} mice showing that Cre (red) is expressed by spinal cord and DRG neurons, but not brain.

(F) *VGLUT3*^{fl/fl}; *Hoxb8*^{Cre} mice lack VGLUT3-IR in the dorsal horn at p10.

(G) VGLUT3-IR is still present in the striatum of *VGLUT3*^{fl/fl}; *Hoxb8*^{Cre} mice.

(H) PWTs of *VGLUT3*^{fl/fl}; *Hoxb8*^{Cre} mice are significantly greater than controls after carrageenan ($n = 11$ and $n = 9$, respectively) and SNI ($n = 9$ and $n = 4$, respectively). Randall-Selitto thresholds are also significantly elevated compared to controls ($n = 9$ and $n = 7$).

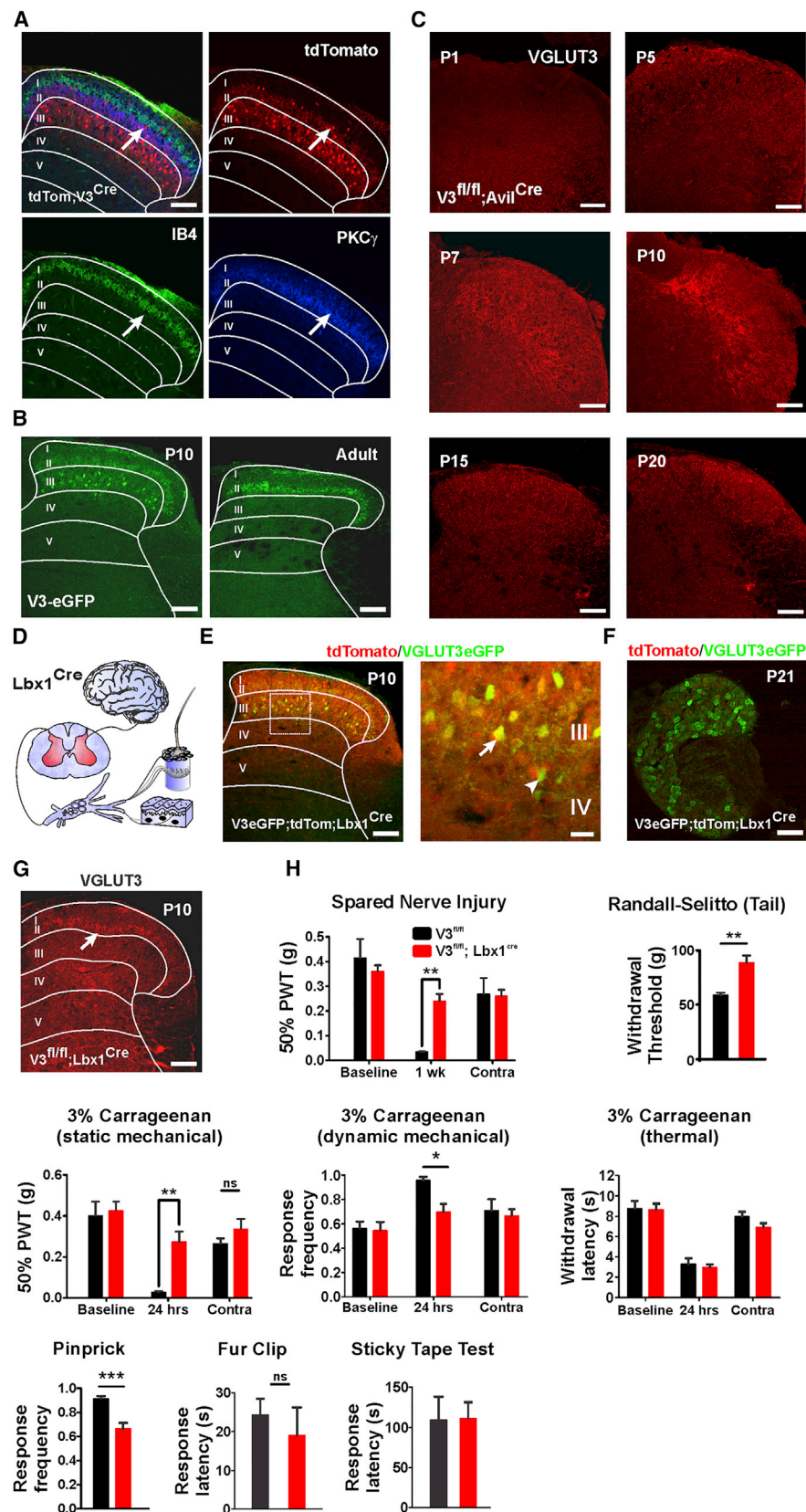
(I) Cartoon of *KRT14*^{Cre} mice showing Cre (red) is restricted to keratinocytes and Merkel cells.

(J) As expected, tomato and the Merkel cell marker TROMA1-IR colocalize (arrowhead) in hindpaw glabrous skin of *Isl*-*tdTomato*; *KRT14*^{Cre} mice.

(K) Tomato and TROMA1-IR also colocalize (arrowheads) in hindpaw glabrous skin of *Isl*-*tdTomato*; *VGLUT3*^{Cre} mice.

(L) PWTs do not differ between *VGLUT3*^{fl/fl}; *KRT14*^{Cre} and control mice before or after carrageenan ($n = 10$ and $n = 6$, respectively) or SNI ($n = 3$, both groups). Randall-Selitto thresholds also do not differ ($n = 7$ and $n = 6$, respectively).

Scale bars, 100 μ m, except in (G) (50 μ m). Data are mean \pm SEM. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.



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peaked around P10–P12, and was significantly reduced by ~P20 (Figure 2C).

To determine whether mechanical pain sensation requires the transient expression of VGLUT3 by dorsal horn neurons, we used *Lbx1^{Cre}* mice. In this line, Cre is restricted to only spinal cord dorsal horn neurons and is not in DRG or brain (Figure 2D) (Sieber et al., 2007). To confirm that the VGLUT3⁺ cells are in the *Lbx1^{Cre}* lineage, we generated VGLUT3^{EGFP};Isl-tdTom;*Lbx1^{Cre}* mice. Virtually all EGFP⁺ cells in the dorsal horn expressed tomato at P10 (Figure 2E). Importantly, no DRG neurons expressed tomato (Figure 2F). Consistent with the deletion of VGLUT3 in the dorsal horn and not in DRG, VGLUT3 immunoreactivity in VGLUT3^{fl/fl};Lbx1^{Cre} mice at P10 appeared only as a discrete band in lamina II, corresponding to C-LTMR afferents (Figure 2G). In the acute mechanical pain assay, tail withdrawal thresholds were significantly elevated compared to VGLUT3^{fl/fl} controls (Figure 2H). Additionally, in the carrageenan and the SNI models of persistent pain, the von Frey thresholds of VGLUT3^{fl/fl};Lbx1^{Cre} mice were significantly higher than control mice after injury. As expected, heat hypersensitivity, which is unaffected in global VGLUT3 KO mice, did not differ from controls. These data confirm that the dorsal horn neurons are the locus of the acute and persistent mechanical pain defects observed in global VGLUT3 KO mice.

To determine in more detail the mechanosensory behaviors that require VGLUT3, we tested the mice in several additional assays. VGLUT3^{fl/fl};Lbx1^{Cre} showed reduced pinprick pain and impaired dynamic mechanical allodynia after carrageenan injection, but showed normal light touch-related behavior in the sticky tape and hair clip assays (Figure 2H).

VGLUT3 Marks a Population of Excitatory Interneurons in the Dorsal Horn

To determine whether VGLUT3 is expressed by inhibitory or excitatory interneurons, we performed double fluorescent in situ hybridizations on Isl-tdTom;VGLUT3^{Cre} mice. Nearly all tomato⁺ cells coexpressed the excitatory (*vglut2*) and not the inhibitory marker (*gad67*) (Figure 3A). Tomato⁺ cells also did not express Pax2, another marker of inhibitory neurons (Figure 3B). To confirm that the mechanical pain defects are due to the loss of VGLUT3 from the excitatory neurons, we generated VGLUT3^{fl/fl};Tlx3^{Cre} mice (Figures 3C–3E), in which Cre is only ex-

pressed by excitatory neurons (Xu et al., 2013). Immunoreactivity for VGLUT3 was absent in the dorsal horn of these mice at P10 (Figure 3D). In all three mechanical pain assays, withdrawal thresholds were significantly elevated compared to VGLUT3^{fl/fl} controls (Figure 3E), consistent with the behavior of global VGLUT3 KO and VGLUT3^{fl/fl};Lbx1^{Cre} mice.

To further identify the transient VGLUT3 neurons, we examined coexpression of tomato with dorsal horn markers in Isl-tdTom;VGLUT3^{Cre} mice (Figures 3F and 3G). At peak VGLUT3 expression (P10), few tomato⁺ cells express PKC γ (16%; 212 of 1,311) or calretinin (10%; 108 of 1,209). Similarly, tomato overlaps with ~25% (212 of 815) of the PKC γ population, and only 7% (108 of 1,469) of calretinin neurons. The degree of overlap is similar in the adult.

Architecture and Afferent Innervation of the VGLUT3 KO Spinal Cord

Gross anatomical defects, such as the aberrant innervation by central terminals of DRG neurons or the loss of spinal cord neurons, have been observed in mouse mutants with spinal cord gene deletions (Ross et al., 2010; Wang et al., 2013; Xu et al., 2013). Such changes could potentially contribute to disruption of the mechanical pain circuit in VGLUT3 KO mice. We therefore examined the distribution of molecular markers for spinal cord neurons (NeuN, vesicular GABA transporter, NK1R, VGLUT2, and PKC γ) and primary afferents (TRPV1, CGRP, isolectin-B4, and VGLUT1) in the dorsal horn of VGLUT3 KO and WT mice (Figure S2A) but observed no major differences. Crossed onto the global VGLUT3 KO strain, Isl-tdTom;VGLUT3^{Cre} mice showed no change in the number of tomato⁺ dorsal horn neurons compared to mice WT for VGLUT3 (Figures S2B and S2C). Thus, deletion of VGLUT3 does not cause apoptosis or obvious changes in the architecture or afferent innervation of the dorsal horn, consistent with a more discrete defect in synaptic transmission.

Synaptic Transmission Defect Is Consistent with Attenuated Mechanical Allodynia

The anatomical basis for light touch becoming painful after injury is thought to involve a disinhibition of the dorsal horn mechanical pain circuits that allows low threshold mechanoreceptors to activate pain transmitting projection neurons in lamina I (NK1R⁺)

Figure 2. Somatosensory Behaviors of VGLUT3^{fl/fl};Lbx1^{Cre} Mice

- (A) In Isl-tdTom;VGLUT3^{Cre} mice, tomato⁺ neurons are located in laminae II–III, and 16% colocalize with PKC γ -IR (arrow).
 (B) In VGLUT3^{EGFP} mice, EGFP is expressed by laminae II–III neurons at p10, but not adult.
 (C) VGLUT3-IR in VGLUT3^{fl/fl};Avil^{Cre} mice peaks around P10 (middle row).
 (D) In *Lbx1^{Cre}* mice, only spinal cord neurons express Cre.
 (E) EGFP and tomato colocalize at p10 in VGLUT3^{EGFP};Isl-tdTom;*Lbx1^{Cre}* mice (arrow). A few EGFP⁺ neurons at the lamina IV border do not express tomato⁺ (arrowhead).
 (F) Tomato is not present in DRG of adult VGLUT3^{EGFP};Isl-tdTom;*Lbx1^{Cre}* mice.
 (G) VGLUT3-IR in VGLUT3^{fl/fl};Lbx1^{Cre} mice at P10 is present in primary afferents (arrow) and not spinal cord neurons.
 (H) Baseline PWT of VGLUT3^{fl/fl};Lbx1^{Cre} mice was similar to controls. After SNI ($n = 8$, both groups), PWTs were significantly elevated compared to controls. Withdrawal responses in the Randall-Selitto assay ($n = 9$, both groups) were also significantly higher than controls. After carrageenan, PWTs of VGLUT3^{fl/fl};Lbx1^{Cre} mice (static mechanical) were elevated compared to controls ($n = 9$, both groups). The mice also responded less frequently than controls to light dynamic mechanical stimulation with a cotton swab ($n = 11$ and $n = 8$, respectively). Latency to respond in the Hargreaves' test did not differ from controls ($n = 11$ and $n = 8$, respectively). VGLUT3^{fl/fl};Lbx1^{Cre} mice responded less frequently than controls to pinprick of the plantar hindpaw ($n = 11$ and $n = 8$, respectively). Sticky tape and fur clip measures of touch did not differ between VGLUT3^{fl/fl};Lbx1^{Cre} mice and controls (both tests $n = 8$ and $n = 7$, respectively). Scale bars, 100 μ m, except inset in (E) (20 μ m). Data are mean \pm SEM. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

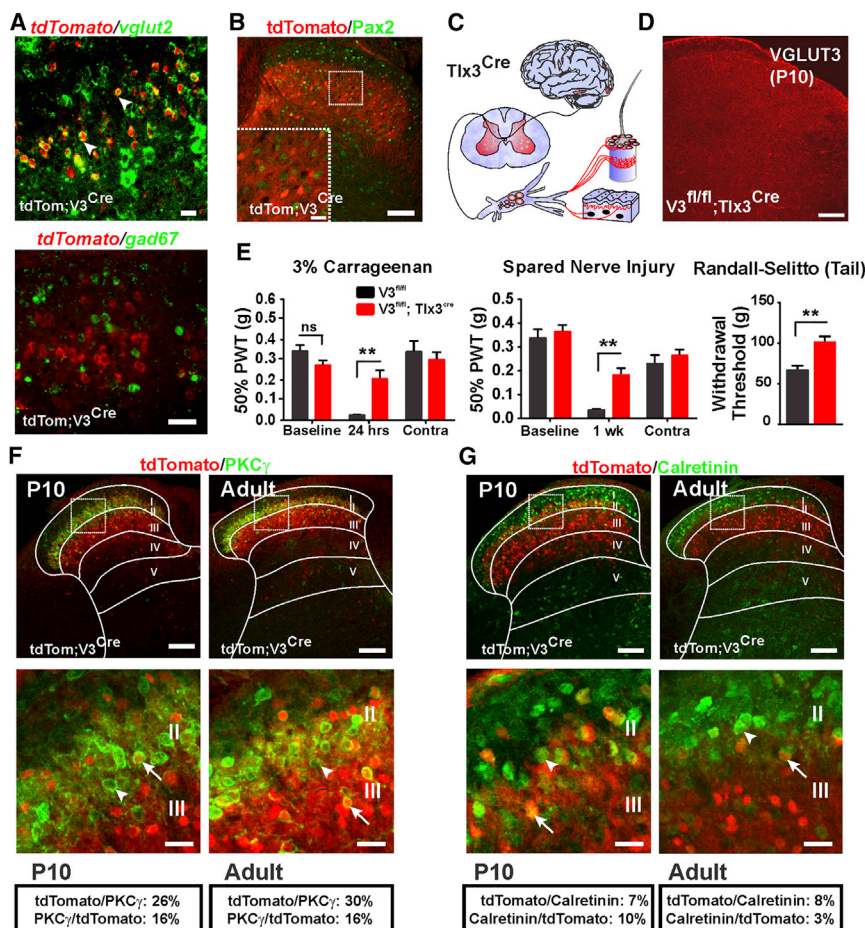


Figure 3. Excitatory Neurons in the Dorsal Horn Transiently Express VGLUT3

(A) In situ hybridization of *tomato* colocalized with *vglut2*, but not *gad67*, in *Isl-tdTom;VGLUT3^{Cre}* mice.

(B) The inhibitory neuron marker *Pax2* does not colocalize with *tomato* in *Isl-tdTom;VGLUT3^{Cre}* mice.

(C) Cartoon showing *Tlx3^{Cre}* mice express *Cre* (red) only in excitatory neurons in the dorsal horn and DRG neurons, and sparsely in brain.

(D) *VGLUT3^{fl/fl};Tlx3^{Cre}* mice at P10 lack *VGLUT3-IR* in the dorsal horn.

(E) Baseline PWTs of *VGLUT3^{fl/fl};Tlx3^{Cre}* mice were similar to controls. After carrageenan ($n = 12$, both groups) or SNI ($n = 11$ and $n = 8$, respectively), PWTs of *VGLUT3^{fl/fl};Tlx3^{Cre}* mice were significantly elevated compared to controls, as were Randall-Selitto tail withdrawal thresholds ($n = 9$ both groups).

(F) Colocalization of *tomato* and *PKCγ* in *Isl-tdTom;VGLUT3^{Cre}* mice at P10 and adult.

(G) Colocalization of *tomato* and *calretinin* in *Isl-tdTom;VGLUT3^{Cre}* mice at p10 and adult.

Scale bars, 100 μ m, except (A) and insets in (B), (F), and (G) (20 μ m). Data are mean \pm SEM. ** $p \leq 0.01$.

through a dorsally directed polysynaptic network (Braz et al., 2014). Since the loss of VGLUT3 from dorsal horn excitatory neurons causes a behavioral impairment in mechanical hypersensitivity, we hypothesized that the low threshold A-fibers would not activate nociceptive lamina I neurons under conditions of disinhibition (the injured state) in the KO mice. To test this hypothesis, we used an in vitro spinal cord model of mechanical allodynia (Torsney and MacDermott, 2006). In this model, when inhibitory tone is normal, dorsal root stimulation at A-fiber intensities is unable to generate polysynaptic excitatory currents in nociceptive lamina I projection neurons. However, under pharmacological disinhibition, stimulation of the dorsal roots at A-fiber intensity reliably generates polysynaptic excitatory currents in the lamina I projection neurons, thus serving as the anatomical substrate for the perception of touch as painful after injury (Baba et al., 2003; Miraucourt et al., 2007; Torsney and MacDermott, 2006). This model is illustrated in Figure 4A.

Whole-cell patch-clamp recordings were performed in transverse spinal cord slices with dorsal roots and DRGs still attached (Figure 4B). Roots were electrically stimulated at different intensities to recruit A β (25 μ A) or A β and A δ (100 μ A) or A β , A δ and C fibers (500 μ A), respectively. To visualize neurons expressing NK1R, slices were preincubated with the fluorescent tetramethylrhodamine substance P conjugate, SP-TMR (Figure 4C), as previously described (Torsney and Mac-

Dermott, 2006). Under normal conditions, A δ -fiber and C-fiber, but not A β -fiber, stimulation produced monosynaptic glutamatergic EPSCs in NK1R⁺ lamina I neurons of both global VGLUT3 KO and control mice, similar to what has been observed in rats (Figure 4D, left traces) (Torsney and MacDermott, 2006). In the presence of the inhibitory receptor antagonists bicuculline (10 μ M) and strychnine (300 nM), stimulation of dorsal roots at A β intensity reliably produced polysynaptic EPSCs in NK1R⁺ lamina I neurons in slices from WT mice (Figure 4D, upper right traces, and Figure 4E). We also occasionally observed A δ -induced polysynaptic EPSCs consistent with previous observations (Figure S2D) (Torsney and MacDermott, 2006). Strikingly, stimulation at A-fiber intensities failed to produce polysynaptic EPSCs in lamina I neurons of VGLUT3 KOs (Figure 4D, bottom right traces and Figure 4E). As a control, increasing the intensity of stimulation to recruit all A-fibers (100 μ A) or all types of fibers (500 μ A) produced monosynaptic EPSCs in the NK1R⁺ neurons similar to those observed in the absence of inhibitory blockers, showing that deletion of VGLUT3 selectively eliminated the polysynaptic EPSCs induced by A β -fibers. Importantly, conduction velocities as well as afferent thresholds measured for A δ - and C-fibers did not differ between VGLUT3 KO and WT mice (A δ threshold, 100 μ A, $p = 0.34$; C threshold, 500 μ A, $p = 0.46$; Figure S2E) and were similar to values previously reported (Torsney and MacDermott, 2006). The results are consistent with a severe impairment in neuronal transmission within the dorsal horn polysynaptic pathway that conveys mechanical hypersensitivity. In addition, the data provide support for altered excitatory transmission rather than a compensatory upregulation of inhibition.

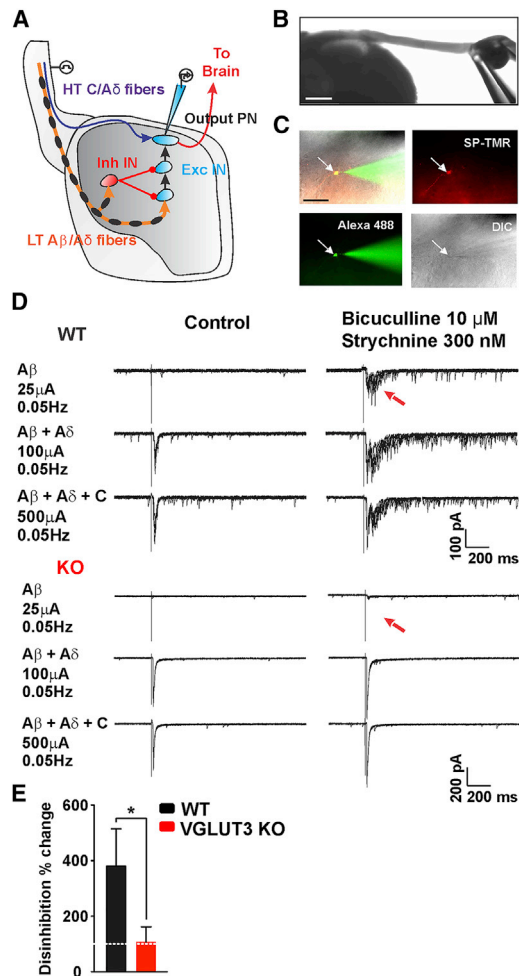


Figure 4. Loss of Polysynaptic Low Threshold Input to NK1R⁺ Lamina I Neurons

(A) Schematic representation of the dorsal horn showing nociceptive inputs (high-threshold [HT] C and A δ fibers) to NK1R⁺ lamina I projection neurons (PN). Polysynaptic innocuous afferent (low threshold [LT] A β and A δ fibers) input to PNs through excitatory interneurons (Exc IN) is suppressed by local inhibitory interneurons (Inh IN). Disinhibition induced pharmacologically enables polysynaptic activation of NK1R⁺ lamina I neurons by LT inputs.

(B) In vitro spinal cord preparation at 4 \times magnification. Scale bar, 500 μ m.

(C) Recorded lamina I NK1R⁺ neuron labeled by fluorescent SP-TMR and filled with Alexa 488. IR-DIC shows same neuron with patch pipette. Scale bar, 100 μ m.

(D) Characterization of afferent inputs to NK1R⁺ lamina I neurons in control conditions (top left traces) and after pharmacological disinhibition (10 μ M bicuculline, 300 nM strychnine) (top right traces). In WT slices, dorsal root stimulation at A δ and C intensities evokes monosynaptic EPSCs in NK1R⁺ lamina I neurons. With pharmacological disinhibition, low-intensity dorsal root stimulation generates A β and A δ polysynaptic EPSCs in NK1R⁺ lamina I neurons. In VGLUT3 KO slices, A δ and C stimulation generates monosynaptic events in NK1R⁺ lamina I neurons (bottom left traces). Under pharmacological disinhibition, low-intensity dorsal root stimulation fails to generate EPSCs in NK1R⁺ lamina I neurons (bottom right traces).

(E) Percent increase in A-fiber-evoked EPSCs in NK1R⁺ lamina I neurons. Data are normalized to EPSCs measured before disinhibition. $n = 7$ (WT) and 8 (VGLUT3 KO) total cells from the same number of mice. Mann-Whitney test, * $p < 0.05$.

VGLUT3⁺ Dorsal Horn Neurons Convey Mechanical Hypersensitivity

Our results show that normal mechanical pain sensation requires the transient postnatal expression of VGLUT3 by dorsal horn neurons, raising the possibility that the neurons themselves convey this type of pain. To test this hypothesis, we used a chemogenetic approach, targeting the excitatory designer receptor (DREADDs) hM3Dq to Cre expressing cells in lamina III of the VGLUT3^{Cre} mice and then assessing whether activation of the cells with the receptor-specific ligand, clozapine-n-oxide (CNO), produces mechanical allodynia. First we verified that the Cre⁺ cells in the dorsal horn of our transgenic line match those responsible for the mechanical pain defects in VGLUT3 KO mice (Figures S3A and S3B). In the Randall-Selitto, carrageenan, and SNI mechanical pain assays, VGLUT3^{fl/fl};VGLUT3^{Cre} mice showed significantly elevated withdrawal thresholds compared to control mice (Figure S3B). Because all adult dorsal horn excitatory neurons express VGLUT2, we predicted that deletion of VGLUT2 from the transient VGLUT3 cells would also produce the mechanical pain defects (Figure S3C). Interestingly, while persistent mechanical hypersensitivity was reduced in the VGLUT2^{fl/fl};VGLUT3^{Cre} mice (similar to knocking out VGLUT3), acute mechanical pain was normal. This unexpected finding is consistent with the idea that although both acute and persistent mechanical pain require the transient postnatal expression of VGLUT3 by these dorsal horn neurons, the cells themselves only transmit persistent pain in the adult.

To target the hM3Dq receptor specifically to the dorsal horn, we used a viral approach. We first determined that the AAV8 serotype coupled with the neuron-specific human synapsin promoter was highly specific for the infection of only dorsal horn neurons (and not DRG) (Figure S4). Three weeks after injection of the AAV8-hSyn-DIO-hM3Dq-mCherry virus into one side of the lumbar enlargement of P10 VGLUT3^{Cre} mice (when Cre is at peak expression), the reporter was highly restricted to neurons in lamina III and importantly was not present in PKC γ neurons or DRG (Figures 5A and 5B). To confirm that hM3Dq functions properly in the dorsal horn, we performed patch-clamp recordings in transverse spinal cord slices. As expected, application of 3 mM CNO produced an inward current in voltage clamp and generated action potentials in current clamp only in mCherry⁺ neurons (Figures 5C and 5D).

To test whether chemogenetic activation of the cells elicits mechanical pain behavior, we measured von Frey thresholds in the presence and absence of CNO (Figure 5E). In the absence of CNO, thresholds for both hindpaws were similar, suggesting that viral infection alone does not affect mechanical sensitivity. After CNO injection, thresholds were dramatically reduced at only the ipsilateral hindpaw, consistent with the induction of mechanical hypersensitivity. The injected mice also showed guarding and occasional fluttering of the ipsilateral hindpaw upon contact with an innocuous surface, indicative of mechanical allodynia (Figure 5F; Movie S1). In a test of heat hypersensitivity (Figure 5E), which is not altered in the global VGLUT3 KO, withdrawal latencies were the same for both hindpaws before and after CNO injection. Since deletion of VGLUT3 in these neurons impairs mechanical hypersensitivity (Figure S3B), we hypothesized that mice lacking the transporter would not show

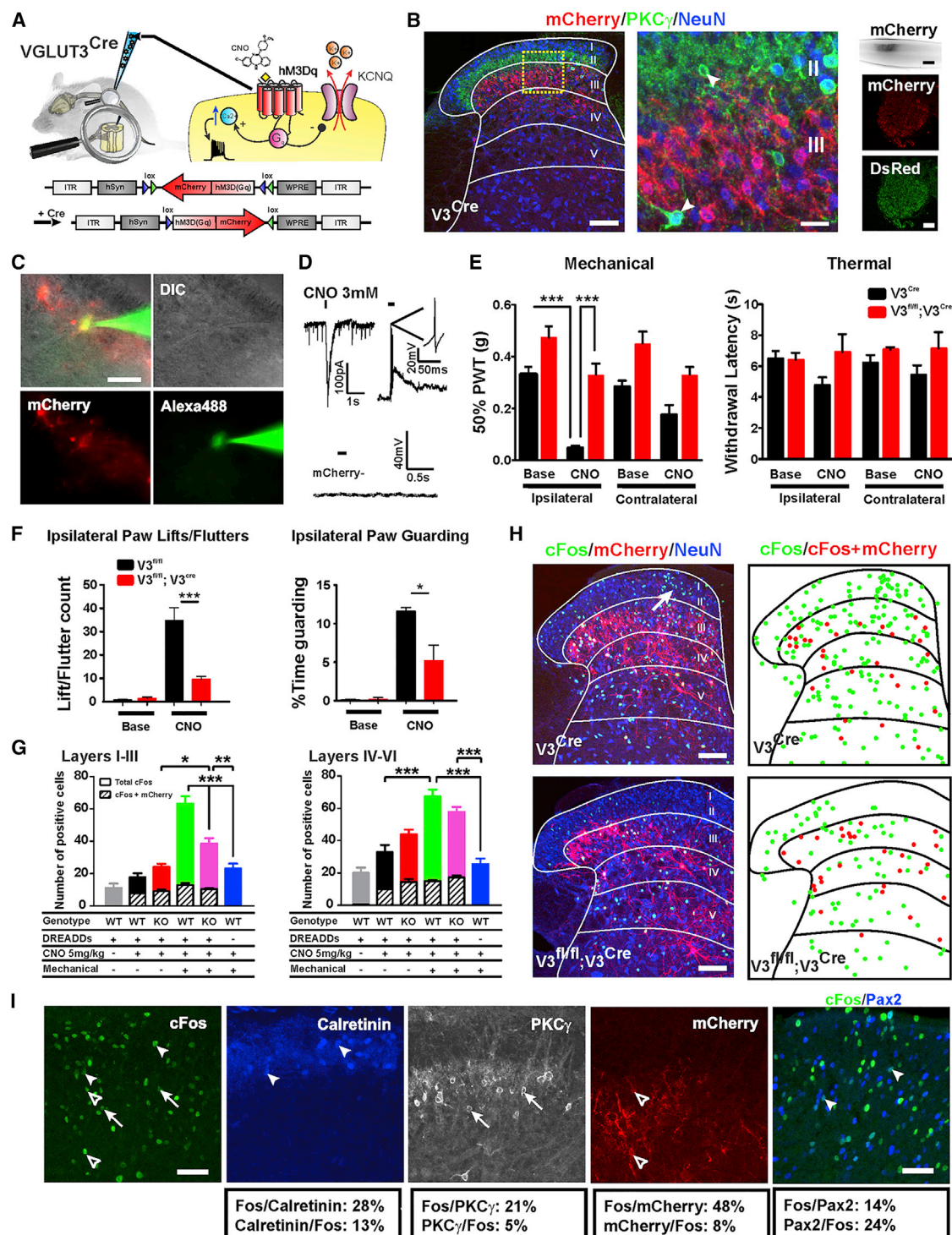


Figure 5. Activation of VGLUT3⁺ Dorsal Horn Neurons Produces Mechanical Allodynia and Reveals a Polysynaptic Mechanical Allodynia Circuit

(A) Schematic of the VGLUT3^{Cre} dorsal horn unilaterally injected at P10 with the Cre-dependent excitatory DREADDs virus (AAV8 hSyn-DIO- hM3Dq-mCherry). (B) Lamina III, but not PKC γ (arrowheads) or DRG, neurons express hM3Dq-mCherry. Scale bars, 100 μ m, except for inset (20 μ m) and whole spinal cord (400 μ m). (C) Recording electrode filled with Alexa 488 targets a lamina III neuron expressing hM3Dq-mCherry. Second electrode used to puff on the specific hM3Dq ligand CNO can be seen in DIC picture. Scale bar, 50 μ m. (D) Direct application of 3 mM CNO to an hM3Dq-mCherry-expressing neuron generates an inward current in voltage clamp and series of action potentials in current clamp. CNO applied to a cell lacking hM3Dq-mCherry does not elicit a change in membrane potential (bottom trace).

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the drop in threshold. Indeed, injection of CNO in VGLUT3^{fl/fl}; VGLUT3^{Cre} mice expressing hM3Dq did not alter the mechanical threshold (Figure 5E). The mice also showed essentially no paw fluttering and significantly less guarding behavior compared to VGLUT3^{Cre} mice (Figure 5F; Movie S1). The data thus provide strong evidence that transient VGLUT3 neurons in lamina III participate in the circuit for mechanical hypersensitivity.

c-Fos Reveals the Dorsally Directed Pathway For Mechanical Hypersensitivity

To identify additional dorsal horn neurons that participate in the mechanical allodynia circuit, we stained for c-Fos, a marker of neuronal activity, after activation of hM3Dq in VGLUT3^{Cre} mice. After injection of CNO in anesthetized mice, c-Fos was surprisingly restricted almost exclusively to the mCherry⁺ cells (Figures 5G and S5). This was also observed with the VGLUT3^{fl/fl};VGLUT3^{Cre} mice. Since mechanical hypersensitivity in the VGLUT3^{Cre} mice requires low threshold input, we followed CNO injection with walking the mice at a pace that was slow, but avoided guarding behavior. Under these conditions, a dramatic increase in c-Fos⁺ cells was observed in laminae I–II and to a lesser degree in lamina III in VGLUT3^{Cre}, but not VGLUT3^{fl/fl};VGLUT3^{Cre} mice (Figures 5G, 5H, and S5). Importantly, c-Fos expression was increased predominantly in the medial dorsal horn, an area corresponding to the distal part of the limb. Mice that did not receive CNO showed little to no c-Fos in the dorsal horn, and mice that received mechanical stimulation, but not CNO, showed c-Fos only in deeper laminae, where innocuous mechanical circuits are active (Figures 5G and S5).

To further identify the c-Fos⁺ neurons in laminae I–III, we costained for PKC γ , calretinin and Pax2. The c-Fos costained with ~48% of mCherry⁺, 21% of PKC γ ⁺, 28% of calretinin⁺, and 14% of Pax2⁺ neurons (Figure 5I). Interestingly, c-Fos was also induced in an excitatory population in lamina III that lacked mCherry and PKC γ . Thus, we have now identified at least four distinct excitatory interneuron populations that reside within the circuit for mechanical hypersensitivity.

To identify neurons postsynaptic to the transient VGLUT3 population in lamina III, we injected a Cre-dependent virus encoding the anterograde trans-neuronal tracer, wheat-germ agglutinin (WGA) (Braz et al., 2002) in Isl-tdTom;VGLUT3^{Cre} mice at P10 (Figure S6). WGA allowed us to refine the position of the neurons with respect to the VGLUT3^{Cre} cells by observing the location of the tracer across time. At the earliest time point (3 dpi), WGA was detected almost exclusively in lamina III

tomato⁺ neurons and not DRG. On days 4 and 5, we detected additional neurons in laminae III and IV as well as vertical cells in lamina II_o, which are all likely to be directly postsynaptic. By 6 and 21 dpi, WGA was still excluded from DRG, but was now detected in inner lamina II where it colocalized with PKC γ and calretinin as well as with inhibitory neurons throughout laminae II–III. We thus conclude that within lamina III, the transient VGLUT3 population is presynaptic to another unidentified population of excitatory neurons, which are presynaptic to lamina II calretinin and PKC γ neurons.

VGLUT3⁺ Lamina III Cells Receive A-Fiber Input

We next determined whether the transient VGLUT3 cells in lamina III directly receive low threshold input, a key component of the dorsal horn circuit for mechanical allodynia (Braz et al., 2014). Using Isl-tdTom;VGLUT3^{Cre} mice, we recorded lamina III tomato⁺ neurons which exhibited equally phasic or tonic firing patterns in response to current injection (Figures 6A and 6B). Remarkably, dorsal root stimulation at A-fiber intensity induced EPSCs in all recorded tomato⁺ neurons (Figures 6C and 6D). About half of the cells (46%) received combined direct monosynaptic A β and polysynaptic A δ inputs and a few received polysynaptic C-fiber input. The high proportion of cells receiving direct A-fiber input, together with the behavioral results, suggest that the transient VGLUT3 population serves as an entry point to the A-fiber-mediated, dorsally directed circuit for mechanical allodynia.

Subsequent morphological analyses revealed that the neurons are fairly homogeneous, showing a predominantly dorso-ventral dendritic arbor. Very few cells have a radial morphology and none resemble central or islet cells (Figure 6E). This finding is consistent with previous reports describing Lamina III excitatory interneurons with dorsally directed processes (Polgár et al., 2007b; Schneider, 2008). Both phasic and tonic neurons showed similar morphologies and afferent input, but phasic neurons were more uniform with only dorsally directed dendrites.

Lamina II Calretinin⁺ Neurons Convey Mechanical Hypersensitivity

Mechanical allodynia resulting from activation of the transient VGLUT3 neurons induced c-Fos expression in calretinin neurons, suggesting that these cells also transmit mechanical hypersensitivity. A small proportion of calretinin cells also transiently express VGLUT3 (Figure 3). We therefore first tested acute and persistent pain behaviors in VGLUT3^{fl/fl};CR^{Cre} mice, but observed no difference compared to VGLUT3^{fl/fl} controls (Figures S7A and S7B).

(E) PWTs for VGLUT3^{Cre} and VGLUT3^{fl/fl};VGLUT3^{Cre} mice expressing hM3Dq-mCherry were normal at baseline. After CNO (5 mg/kg) injection, only PWTs of VGLUT3^{Cre} mice dropped dramatically. Hargreaves' withdrawal latencies were unchanged for the two mouse lines (both tests $n = 7$ and $n = 10$, respectively). (F) After CNO (5 mg/kg) injection, hM3Dq-expressing VGLUT3^{Cre} mice show lifting, fluttering, and guarding of the ipsilateral hindpaw. In contrast, hM3Dq-expressing VGLUT3^{fl/fl};VGLUT3^{Cre} mice rarely exhibit the behaviors.

(G) Quantification of c-Fos⁺ cells in laminae I–III and laminae IV–VI of VGLUT3^{Cre} (labeled WT) and VGLUT3^{fl/fl};VGLUT3^{Cre} (labeled KO) mice expressing hM3Dq-mCherry. Hatched bars indicate number of c-Fos⁺ cells that also express mCherry for each condition.

(H) Induction of c-Fos cells in laminae I–III neurons of VGLUT3^{Cre}, but not VGLUT3^{fl/fl};VGLUT3^{Cre}, mice (both expressing hM3Dq-mCherry) after CNO injection followed by mechanical stimulation. Scale bars, 100 μ m.

(I) Costaining of c-Fos with PKC γ (21%), calretinin (28%), and Pax2 (14%) neurons in laminae I–III of VGLUT3^{Cre} mice expressing hM3Dq-mCherry after CNO injection followed by mechanical stimulation. Nearly half (48%) of the mCherry⁺ cells were also c-Fos⁺. Scale bars, 50 μ m.

Data are mean \pm SEM. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

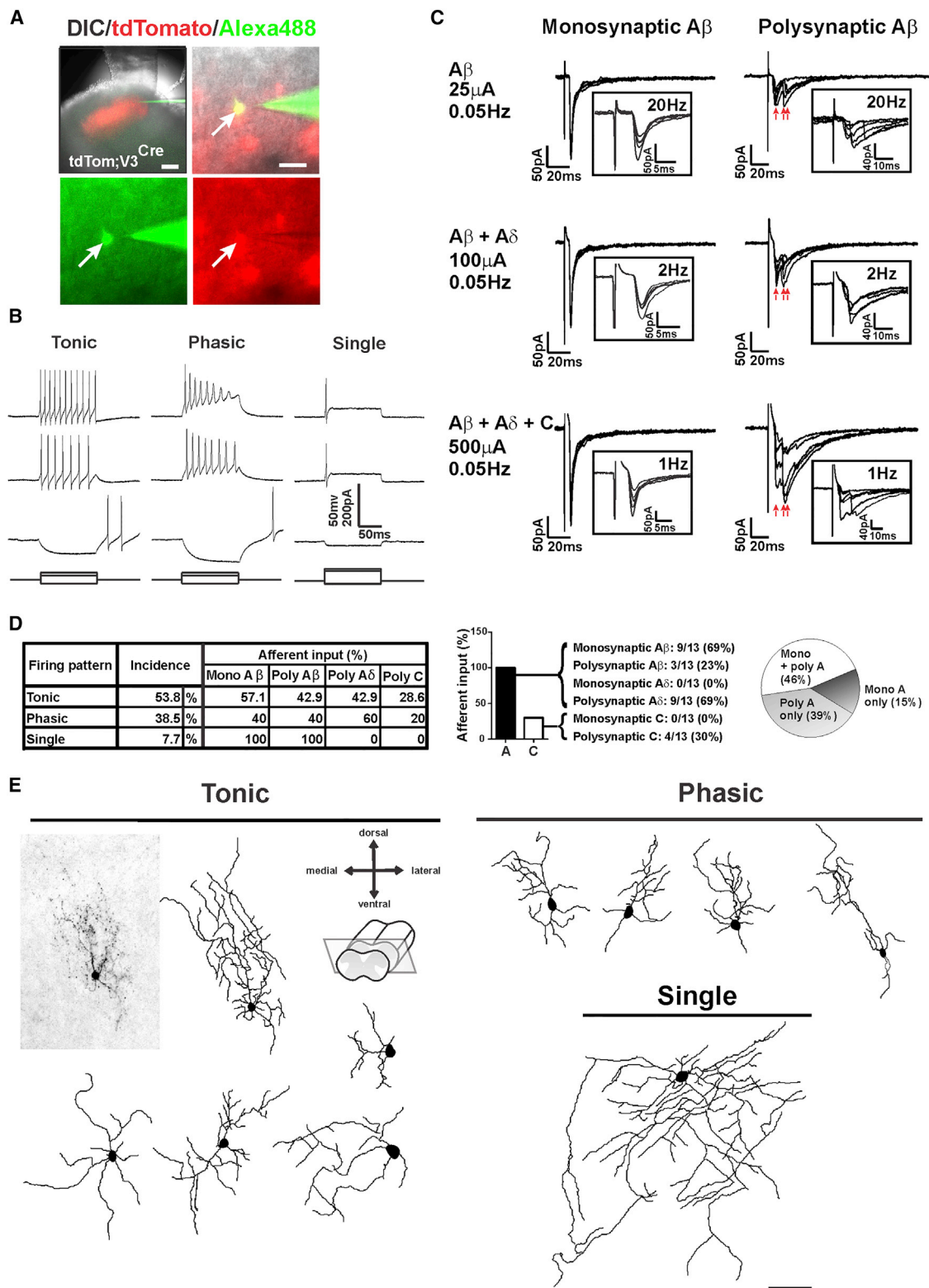


Figure 6. Characterization of VGLUT3⁺ Neurons and Primary Afferent Input

(A) Lsl-tdTom;VGLUT3^{Cre} neurons were recorded using transverse spinal cord slices with the dorsal root and DRG still attached. Cells were filled with Alexa 488 and biotin for post hoc reconstruction. Scale bars, 200 μ m (upper left) and 50 μ m.

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To directly manipulate calretinin neurons, we injected the hM3Dq virus into the dorsal horn of CR^{Cre} mice (Figure 7A). Expression of the mCherry was limited to a discrete layer of calretinin⁺ cells within the dorsal part of lamina II_i and was absent from PKC γ and DRG neurons (Figures 7B, S7C, and S7D). Importantly, mCherry expression corresponded to calretinin⁺ cells in the adult and not to neurons in lamina I, II_o, and the ventral part of lamina II that transiently express calretinin during development and can be observed in Isl-tdTom;CR^{Cre} mice (Figure S7E). These adult calretinin neurons are largely distinct from an Lbx1⁺ population (Figure S7F) recently shown to affect acute pain (Duan et al., 2014). After CNO injection, the ipsilateral von Frey threshold dropped dramatically (Figure 7D). Behaviors such as paw guarding and fluttering were also observed in the ipsilateral paw (Figure 7E; Movie S2), consistent with mechanical hypersensitivity and similar to what we observed with VGLUT3^{Cre} mice. Thermal sensitivity in these mice was unchanged (Figure 7D).

We also examined the dorsal horn expression of c-Fos in these mice. In anesthetized animals injected with CNO, c-Fos was induced primarily in mCherry⁺ cells (Figures 7F, 7G, and S7G). However, coupling low threshold mechanical stimulation with CNO dramatically increased the number of c-Fos neurons in laminae I–II (Figures 7F, 7G, and S7H). This increase was absent in mice lacking hM3Dq or injected with saline (Figure 7G).

To further characterize the c-Fos⁺ cells, we costained for calretinin, PKC γ , and Pax2 (Figure 7H). Approximately 30% of the calretinin neurons expressed mCherry, and half of these showed c-Fos induction. Of the remaining mCherry-negative calretinin neurons, ~45% expressed c-Fos, indicating a high degree of activation within the calretinin population. We also observed ~30% of c-Fos⁺ cells costained with Pax2. Most interestingly, very few of the c-Fos⁺ cells costained for PKC γ .

Our data thus far show that hM3Dq activation of either the transient VGLUT3 neurons in lamina III or the adult calretinin neurons in lamina II produces mechanical hypersensitivity, and that coupling a mechanical stimulus with the activation of these cells induces c-Fos in the dorsal horn circuit for mechanical allodynia. Interestingly, activation of the transient VGLUT3, but not the calretinin neurons, produces c-Fos in PKC γ neurons, suggesting the existence of more than one allodynic pathway. Since the discovery of PKC γ as a critical component of persistent mechanical pain (Malmberg et al., 1997), evidence for its contribution to allodynia produced by different types of injury has remained unclear (Gao and Ji, 2010; Zhao et al., 2011; Zou et al., 2011). We therefore compared the pattern of c-Fos induction in the carrageenan and SNI models (Figures 8A–8F).

Lsl-tdTom;VGLUT3^{Cre} mice were injected with carrageenan with or without low threshold mechanical stimulation 24 hr later. As expected, the number of c-Fos⁺ cells in laminae I–III was increased dramatically in the mice that also received mechanical stimulation (Figures 8A and 8B). The distribution of c-Fos was strikingly similar to what was observed by activating the calreti-

nin population together with mechanical stimulation, including a number of calretinin (~17%) and Pax2 (~41%) cells but very few PKC γ neurons (~1%) (Figure 7H). Additionally, a significant number of c-Fos⁺ cells coexpressed tomato (16%), indicating activation of the transient VGLUT3 population.

For the SNI model, mice that received mechanical stimulation three days after surgery showed a substantial increase in c-Fos⁺ cells in laminae I–II (Figures 8D and 8E). In contrast to the inflammatory pain model, a significant number of PKC γ (11%) and fewer calretinin (8%) neurons expressed c-Fos (Figure 8F). Again, a substantial number of c-Fos⁺ cells coexpressed tomato (~11%). Together, our data show that distinct spinal circuits can lead to mechanical allodynia, with both requiring activation of the transient VGLUT3 neurons.

DISCUSSION

This study shows that the critical role of VGLUT3 in mechanical pain arises from its transient expression by dorsal horn excitatory interneurons. The cells directly receive A β primary sensory input and are required for polysynaptic transmission onto lamina I pain processing neurons after injury. Chemogenetic activation of transient VGLUT3 neurons in lamina III induces mechanical allodynia in a circuit that also includes PKC γ and calretinin cells in lamina II. Activation of calretinin neurons also induces mechanical hypersensitivity and engages a distinct, dorsally directed pathway that does not include the PKC γ neurons. Further, we show that mechanical allodynia observed in models of inflammation and neuropathy is mediated by distinct neural circuits. Both require the participation of transient VGLUT3 neurons but a distinct complement of second-order neurons.

Role of VGLUT3 in the Somatosensory System

Although VGLUT3 expression in the dorsal horn of adult mice is restricted to C-LTMRs, we now show that loss of the transporter from this population does not produce the mechanical pain defects observed in global VGLUT3 KO mice. Using a conditional VGLUT3 KO and several Cre-driver lines, including the spinal cord-specific driver Lbx1^{Cre}, we have determined that the defects are due to the loss of VGLUT3 from spinal cord neurons that express the transporter transiently during postnatal development. The finding, however, does not address whether the C-LTMRs themselves are important for pain. These DRG neurons also express VGLUT2 and tyrosine hydroxylase, which may provide alternative mechanisms for signaling (Li et al., 2011; Scherrer et al., 2010). Studies in humans and rodents suggest C-tactile afferents transmit pleasant touch sensation under normal conditions (Löken et al., 2009; Vrontou et al., 2013) and may have a role in suppressing pain potentially by activating islet cells (but also see Nagi et al.) (Delfini et al., 2013; Liljencrantz et al., 2013; Nagi et al., 2011). Consistent with this idea, the C-LTMRs do not contact the pain-promoting PKC γ neurons that also reside in this layer (Peirs et al., 2014).

(B) Example traces of tonic, phasic, and single firing patterns recorded in tomato⁺ neurons.

(C) The tomato⁺ neurons receive monosynaptic or polysynaptic A β inputs.

(D) Incidence of firing patterns and afferent inputs of tomato⁺ neurons.

(E) Morphology of lamina III tomato⁺ neurons. Scale bar, 50 μ m.

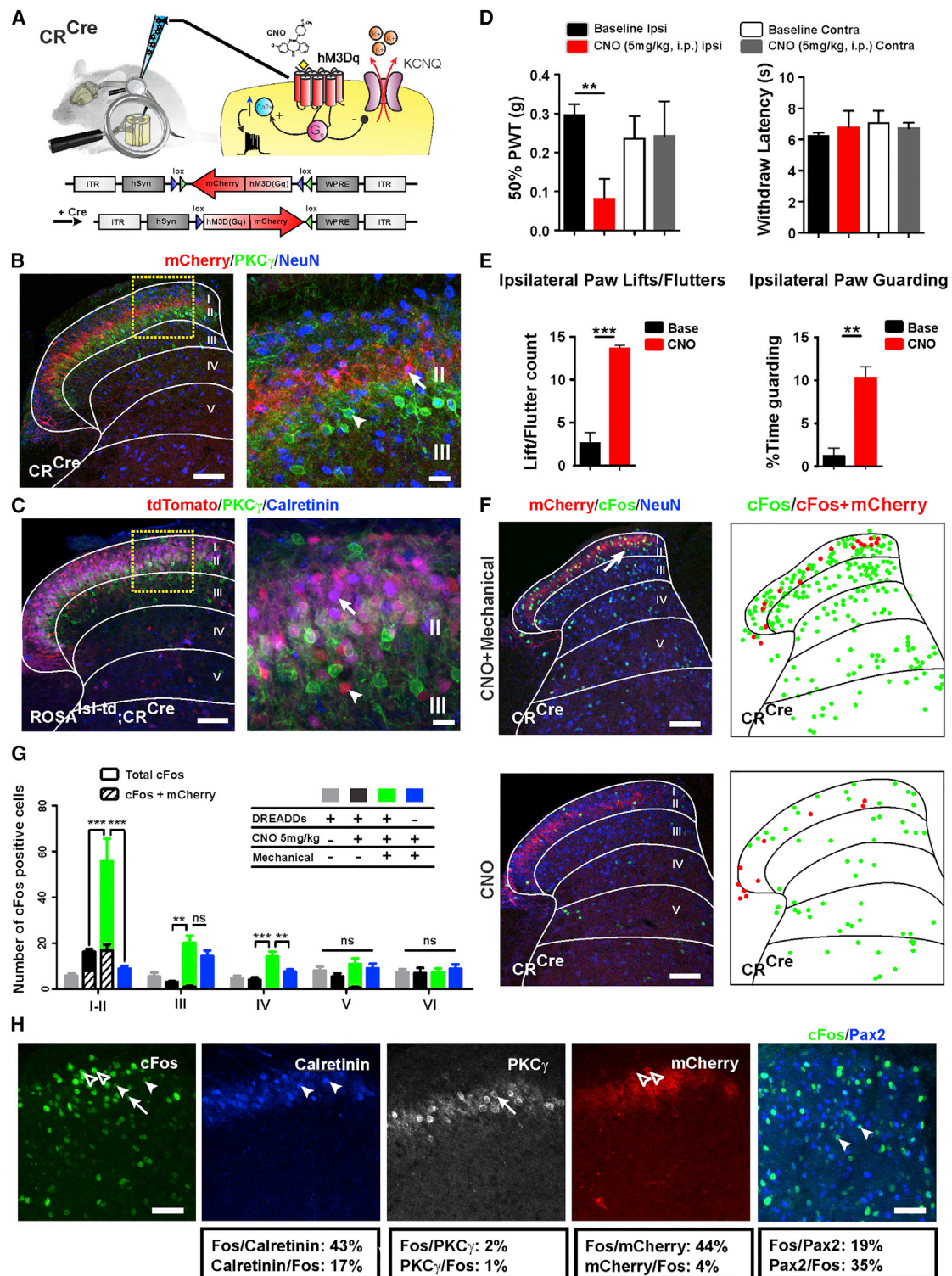


Figure 7. Activation of Calretinin⁺ Neurons Produces Mechanical Allodynia

(A) Schematic of the CR^{Cre} dorsal horn unilaterally injected with the hM3Dq-mCherry virus.

(B) The mCherry localizes exclusively to the dorsal region of lamina II_i (arrow), and does not colocalize with PKC γ (arrowhead). Scale bars, 100 μ m (left) and 20 μ m (right).

(C) In Isl-tdTom;CR^{Cre} mice, tomato is expressed by neurons throughout lamina II_i and costains almost completely with calretinin, but very little with PKC γ . Scale bars, 100 μ m (left) and 20 μ m (right).

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Our analysis of the dorsal horn architecture, including the pattern of primary afferent innervation and electrophysiological properties, showed no evidence of gross abnormalities in the VGLUT3 KO mice. However, using an *in vitro* model of mechanical allodynia, we find that low threshold A-fibers are unable to activate NK1R⁺ lamina I neurons when inhibitory transmission in the dorsal horn is completely blocked, indicating that the loss of mechanical hypersensitivity reflects a profound impairment in excitatory transmission between second-order neurons. Interestingly, a small decrease in excitatory transmission in this polysynaptic pathway, such as blocking only NMDA receptors, can also prevent the activation of lamina I neurons (Torsney and MacDermott, 2006). Thus, a local change in synaptic transmission due to the absence of VGLUT3 could have a dramatic downstream impact. Importantly, the transient expression of VGLUT3 coincides with a critical period in the maturation of the dorsal horn, a time when the interneurons are establishing their mature firing patterns and connectivity (Fitzgerald, 2005). At the synaptic level, both NMDA and AMPA receptors are undergoing a series of changes, including the awakening of silent synapses and activity-dependent alterations in subunit composition (Baba et al., 2000; Li and Zhuo, 1998). Precisely how VGLUT3 participates in these or other developmental changes to properly set up the mechanical pain circuit remains unclear and the subject of further investigation.

Spinal Cord Circuit for Mechanical Hypersensitivity

Original models of the dorsal horn circuit for mechanical allodynia have focused almost exclusively on a series of synaptically connected neurons that transmit touch information to superficial layers (Lu et al., 2013; Miraucourt et al., 2007; Torsney and MacDermott, 2006). This serial circuit starts with PKC γ neurons, which then activate central cells in lamina II_i, vertical cells in lamina II_o, and finally the lamina I projection neurons (Lu et al., 2013; Lu and Perl, 2005). More recently, additional circuits have been proposed (Braz et al., 2014; Yasaka et al., 2014) to support the idea that the induction and transmission of mechanical hypersensitivity within the dorsal horn involve integrating information through multiple mechanisms and neuronal networks.

Surprisingly, deep dorsal horn neurons, which receive the major input from low threshold sensory neurons, have been largely ignored. We now show using a chemogenetic approach that the transient VGLUT3 neurons located in lamina III directly participate in the mechanical allodynia circuit. Interestingly, although loss of VGLUT3 from the laminae II–III dorsal horn neurons attenuates both acute and persistent pain, deletion of VGLUT2, the transporter that mediates glutamate signaling by these neurons

in the adult, produces defects only in persistent mechanical pain, suggesting that the neurons themselves do not convey acute mechanical pain. This result is consistent with the lack of acute pain behavior (biting and licking) observed when we activate hM3Dq in the VGLUT3^{Cre} mice. The paw guarding and fluttering behavior that we do observe in these mice is more consistent with the actions of a limb sensitive to touch.

We also now report that activation of calretinin neurons in lamina II of adult mice specifically induces mechanical allodynia. These neurons are distinct from the calretinin population recently shown to be important only for light mechanical pain (Duan et al., 2014). Thus, there are at least two distinct calretinin populations in lamina II, one that transiently expresses calretinin together with Lbx1 and is not involved in mechanical hypersensitivity, and a second population that permanently expresses calretinin, but never Lbx1, and is important for persistent mechanical pain. Additionally, the adult calretinin population and the transient VGLUT3 population are distinct from the somatostatin⁺ excitatory neurons in lamina II that express Lbx1 and partially overlap with PKC γ neurons and likely vertical cells (Duan et al., 2014; Polgár et al., 1999) and were recently implicated in persistent mechanical pain.

The transient VGLUT3 cells in lamina III receive input from myelinated A β fibers, placing them at an entry point for the dorsally directed mechanical allodynia circuit. The anterograde tracing and c-Fos results provide a map of the rest of the allodynia circuit starting from these lamina III transient VGLUT3 neurons. Interestingly, we find that the neurons are connected to a second population of excitatory interneurons in this same lamina. These latter, still-unidentified neurons likely serve as the connection between lamina III and the more dorsally located neurons in the pathway, including PKC γ and calretinin neurons. The tracing study also shows a direct connection between the transient VGLUT3 neurons and cells in lamina II_o, which are likely to be vertical cells because unlike other neurons in lamina II, these cells have long ventrally directed dendrites that reach into lamina III. Consistent with our observation, Kato et al. identified a population of lamina III excitatory neurons that provides a major input to the lamina II vertical cells and thus a potentially shorter route to the nociceptive projection neurons (Kato et al., 2009).

It has been suggested that the dorsal horn circuit for mechanical allodynia is controlled by a feedforward inhibition evoked by low threshold inputs that prevent touch from becoming painful (Braz et al., 2014; Zeilhofer et al., 2012). This concept has elicited much interest, as it is the central element of “the gate control theory of pain” published by Melzack and Wall in 1965. In this

(D) PWTs of CR^{Cre} mice expressing hM3Dq-mCherry were normal at baseline, but dropped dramatically after injection of CNO (5 mg/kg). Hargreaves' withdrawal latencies were unaffected ($n = 8$ for both tests).

(E) After CNO (5 mg/kg) injection, CR^{Cre} mice expressing hM3Dq-mCherry demonstrate lifting, fluttering, and guarding of the ipsilateral paw.

(F) Representative images and schematics of c-Fos in the dorsal horn of CR^{Cre} mice expressing hM3Dq-mCherry after CNO only (top) or CNO followed by mechanical stimulation (bottom). Scale bars, 100 μ m.

(G) Quantification of c-Fos⁺ neurons in laminae I–II and laminae III–IV after CNO injection with or without mechanical stimulation, mechanical stimulation only or saline injection only.

(H) c-Fos⁺ neurons in laminae I–II induced by CNO plus mechanical stimulation costain with calretinin (17%) and Pax2 (35%), but much less with PKC γ (1%). Nearly half (44%) of the mCherry⁺ cells colocalized with c-Fos. Scale bars, 50 μ m.

Data are mean \pm SEM. ** $p \leq 0.01$, *** $p \leq 0.001$.

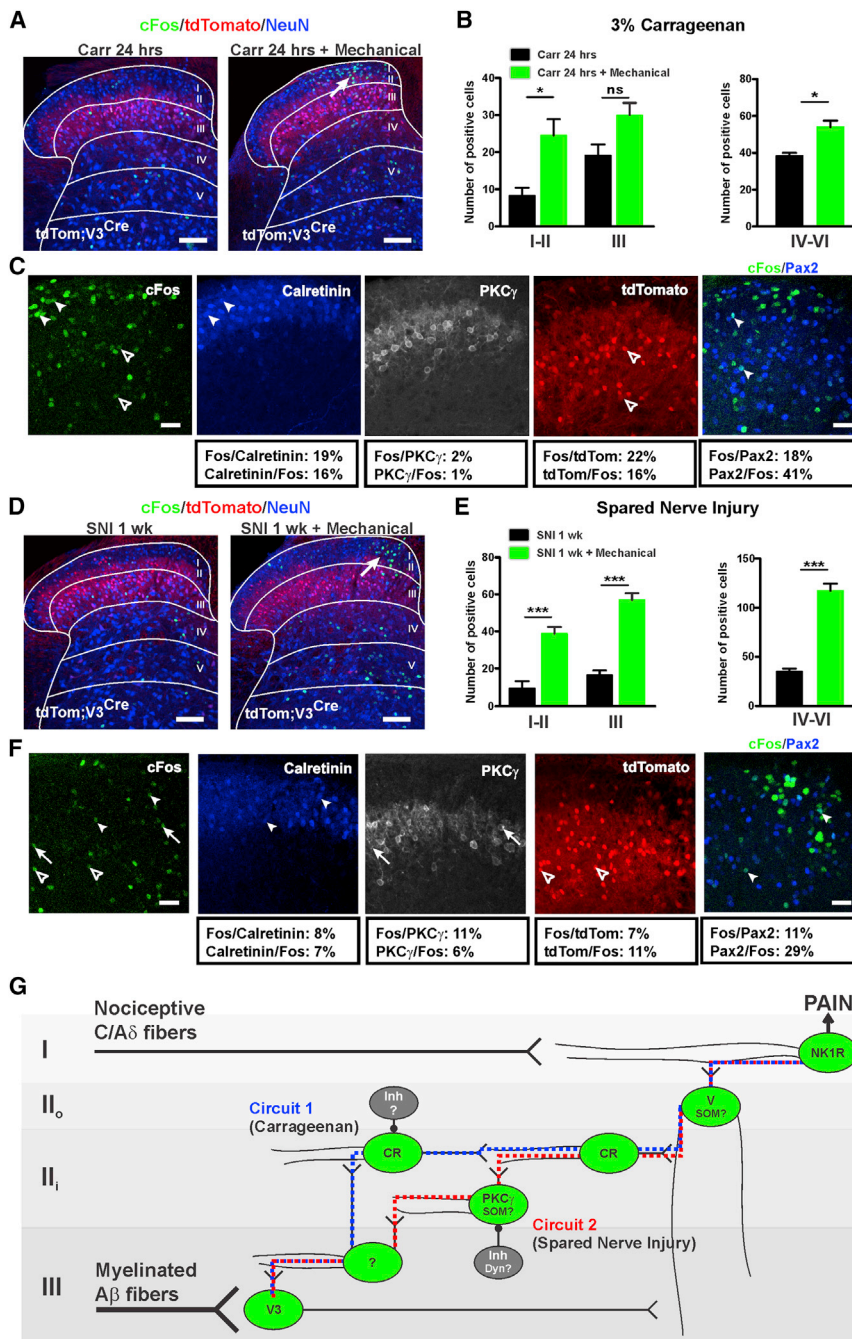


Figure 8. Expression of c-Fos in Inflammatory and Neuropathic Pain Models Reveals Multiple Microcircuits for Mechanical Allodynia

(A) Representative pictures of c-Fos induced by carrageenan injection and with (right) or without (left) mechanical stimulation 24 hr later. Scale bars, 100 μ m.

(B) Quantification of c-Fos⁺ dorsal horn cells after carrageenan injection and with (green bars) or without (black bars) mechanical stimulation 24 hr later.

(C) c-Fos⁺ cells colocalize with calretinin (16%), very few with PKC γ (1%), as well as tomato⁺ lamina III neurons (16%) and Pax2⁺ cells (41%). Scale bars, 50 μ m.

(D) Representative pictures of c-Fos induced by SNI with (right) or without (left) mechanical stimulation delivered 1 week later. Scale bars, 100 μ m.

(E) Quantification of c-Fos⁺ dorsal horn cells after SNI with (green bars) or without (black bars) mechanical stimulation delivered 1 week later.

(F) c-Fos colocalizes with cells expressing calretinin (7%), PKC γ (6%), tomato (11%), and Pax2 (29%). Scale bars, 50 μ m.

(G) Schematic diagram of the circuit for mechanical allodynia. Transient VGLUT3 cells located in lamina III transmit the input from myelinated fibers to the more dorsal neural network underlying mechanical hypersensitivity. Neighboring cells in lamina III receive input from transient VGLUT3 neurons and relay the signal to lamina II cells including PKC γ ⁺ and calretinin⁺ excitatory interneurons that act to refine the excitability of the circuit. Vertical cells in lamina II_o, which receive input from transient VGLUT3 cells and lamina II cells, integrate the signal and send an output to the nociceptive NK1R⁺ lamina I projection neurons. In persistent pain induced by nerve injury, the “gate” that controls PKC γ is opened, resulting in activation of the VGLUT3-PKC γ -calretinin-vertical cell-NK1R pathway (red dotted line). During inflammation, the “gate” that controls calretinin neurons is opened, resulting in the activation of the VGLUT3-calretinin-vertical cell-NK1R pathway (blue dotted line). V3, transient VGLUT3; SOM, somatostatin; CR, calretinin; Inh, inhibitory interneurons; Dyn, dynorphin; V, vertical; NK1R, neurokinin 1 receptor.

Data are mean \pm SEM. * p < 0.05, *** p \leq 0.001.

model, revisited in recent reviews (Braz et al., 2014; Zeilhofer et al., 2012), the “gate” represents a population of inhibitory interneurons that facilitate nociceptive circuits and also prevent the passage of touch-related information from deep neurons to more superficial pain-processing cells. PKC γ -expressing neurons reside at the border between touch and pain (lamina II) and have been suggested to be key elements of the circuit (Lu et al., 2013; Miraucourt et al., 2007). The cells have been modeled as initiating the activation of a dorsally directed pathway transforming touch into pain when the “gate is opened,” i.e., when

the inhibition controlling their activity is decreased by injury. Our data now refine how these neurons participate in mechanical allodynia. First, we now show that neurons more ventrally located than the PKC γ neurons are required to elicit mechanical pain behavior in the carrageenan and SNI models as well as to evoke EPSCs in lamina I in the *in vitro* assay of mechanical allodynia. Interestingly, these transient VGLUT3 neurons do not show evidence of being under significant feedforward inhibition, as they reliably exhibit evoked EPSCs and membrane depolarization upon dorsal root stimulation. Thus, although these

neurons are likely to engage the pathway, the nexus of inhibition must instead be on the postsynaptic lamina III neurons or the excitatory populations in lamina II (Duan et al., 2014), including the PKC γ neurons, which are known to be under strong inhibitory control (Lu et al., 2013). Second, we observed c-Fos expression in PKC γ neurons in the SNI model of neuropathic pain, but not the carrageenan model of inflammatory pain, suggesting that the involvement of these neurons in the two models of mechanical hypersensitivity may differ. The precise role of PKC γ neurons in different types of injury will require further investigation. From this work, we now propose that the mechanical allodynia circuit is composed of multiple overlapping microcircuits with distinct gates that are engaged by different types of injuries (see model in Figure 8). The more comprehensive view of the spinal cord circuitry provided by this study will help guide efforts to design and implement new treatment strategies.

EXPERIMENTAL PROCEDURES

Animals

Mice were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Conditional VGLUT3 KO mice (VGLUT3^{fl/fl}) were crossed to the Rosa26^{Cre} line to generate VGLUT3^{Δ/Δ} and to Advillin^{Cre}, Hoxb8^{Cre}, KRT14^{Cre}, Tlx3^{Cre}, Lbx1^{Cre}, SNS^{Cre}, or VGLUT3^{Cre} to generate cell-specific deletions. Detailed description of VGLUT3^{fl/fl} and other mouse lines can be found in Supplemental Experimental Procedures.

Surgical Procedures

SNI was performed as previously described. Spinal cord injections of AAV were performed at P9–P10 or P15–P16. Under isoflurane, midline incision was made without laminectomy and virus delivered (1 μ l) slowly with a glass microelectrode (50 μ m tip) between lumbar segments L4 and L5. Silk sutures were used to close *lassimus dorsi*, and skin and Ketofen was given before and 1 day after surgery. Behavior was tested 3 weeks later. For more details including virus titer, see Supplemental Experimental Procedures.

Electrophysiological Recordings and Morphology

Transverse slices were made from lumbar spinal cord of P25–P35 mice, keeping roots and DRG attached. Whole-cell patch-clamp recordings were made with neurobiotin in the pipette when morphology was also performed. For details, see Supplemental Experimental Procedures.

In Situ Hybridization and Immunohistochemistry

ISH and IHC were performed as previously described (Seal et al., 2009). Induction of c-Fos was performed under urethane anesthetic or with mechanical stimulation by slowly walking mice on a treadmill (10 cm/s). Mice were perfused with 4% paraformaldehyde 90 min after the treatment (see Supplemental Experimental Procedures).

Statistics

Data are reported as mean \pm SEM. Randall-Selitto, pinprick, sticky tape, and fur clip were analyzed by two-tailed Student's *t* test. Von Frey, Hargreaves', and spontaneous behavior were analyzed by two-way ANOVA with Bonferroni's post hoc test. Significance was considered *p* < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental information includes seven figures, two movies, and Supplemental Experimental Procedures and can be found with this article at <http://dx.doi.org/10.1016/j.neuron.2015.07.029>.

AUTHOR CONTRIBUTIONS

C.P., S.-P.G.W., X.Z., and R.P.S. designed experiments; C.P., S.-P.G.W., X.Z., C.E.W., J.Y.G., N.E.C., A.C.G., Z.L., and P.S.M. performed experiments; H.H. provided a reagent; C.P., S.-P.G.W., X.Z., and R.P.S. analyzed data; and C.P., S.-P.G.W., and R.P.S. wrote the paper.

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